Stem Cell Research (2011) 6, 34–49

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REGULAR ARTICLE

One-step generation of murine embryonic stem cell-derived mesoderm progenitors and chondrocytes in a serum-free monolayer differentiation system

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Received 22 March 2010; received in revised form 23 August 2010; accepted 27 August 2010

Abstract Cartilage defects have limited capacity for repair and are often replaced by fibrocartilage with inferior mechanical properties. To overcome the limitations of artificial joint replacement, high-throughput screens (HTS) could be developed to identify molecules that stimulate differentiation and/or proliferation of articular cartilage for drug therapy or tissue engineering. Currently embryonic stem cells (ESCs) can differentiate into articular cartilage by forming aggregates (embryoid body (EB), pellet, micromass), which are difficult to image. We present a novel, single-step method of generating murine ESC-derived chondrocytes in monolayer cultures under chemically defined conditions. Mesoderm induction was achieved in cultures supplemented with BMP4, activin A, or Wnt3a. Prolonged culture with sustained activin A, TGFβ3, or BMP4 supplementation led to robust chondrogenic induction. A short pulse of activin A or BMP4 also induced chondrogenesis efficiently while Wnt3a acted as a later inducer. Long-term supplementation with activin A or with activin A followed by TGFβ3 promoted articular cartilage formation. Thus, we devised a serum-free (SF) culture system to generate ESC-derived chondrocytes without the establishment of 3D cultures or the aid of cell sorting. Cultures were governed by the same signaling pathways as 3D ESC differentiation systems and limb bud mesenchyme or articular cartilage explant cultures.

Introduction

Articular cartilage of the vertebrate skeleton consists of chondrocytes suspended in rigid extracellular matrix (ECM) and provides a resilient barrier between bones while facilitating load-bearing and joint articulations. Articular cartilage damages are triggered by pathological degradation from enzymes and inflammatory cues in osteoarthritis and rheumatoid arthritis, or they can be caused by physical trauma like intraarticular fractures and ligament injuries (Beris et al., 2005). The avascularity and low metabolic rate of articular cartilage limit the repair capacity of partial-thickness defects due to the inability of progenitor cells to travel through the ECM to the injury site (Vinatier et al., 2009; van Osch et al., 2009). The search for new therapeutic approaches has led to the use of ESCs as a potential renewable chondrogenic cell source in standardized platforms for novel drug screens or cellular therapy for articular cartilage repair. Therefore, it is imperative to fully understand the molecular mechanisms governing germ layer induction and tissue patterning to efficiently generate ESC-derived chondrocytes.

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doi:10.1016/j.scr.2010.08.007

During gastrulation, the first mesodermal cells including hematopoietic progenitors emerge from the posterior part of the early primitive streak migrating anteriorly and laterally. Cardiac, cranial, and lateral plate mesoderm progenitors are formed during the mid-streak stage, followed by the ingression of progenitors that specify trunk and paraxial mesoderm (muscle, bone, and cartilage precursors) in the late-streak stage (Loebel et al., 2003; Kinder et al., 2001). Cartilage formation during endochondral bone development is initiated by mesenchymal condensation, within which cells differentiate into chondrocytes that express the transcription factor *SRY (sex-determining region Y)-box 9* (*Sox9*) (Lefebvre et al., 1997; Bi et al., 1999; Akiyama et al., 2002; Lefebvre and Smits, 2005), which regulates the production of type II collagen (Col2a1) and the proteoglycan Aggrecan (Goldring et al., 2006; Poole et al., 2001). *Proteoglycan-4* (*PRG4*) encodes a superficial zone protein synthesized specifically by articular cartilage (Schumacher et al., 1999; Flannery et al., 1999). Chondrocytes undergo hypertrophy prior to matrix calcification (Olsen et al., 2000; Provot and Schipani, 2005; de Crombrugghe et al., 2001), producing type X collagen (Col10a) and expressing the master regulator of osteoblast differentiation *runt-related transcription factor 2* (*Runx2*) (Karsenty and Wagner, 2002; Otto et al., 1997; Komori et al., 1997).

ESC models of primitive streak formation and lineage commitment validate the possibility of generating functional cell types under defined conditions (Gadue et al., 2006; Kattman et al., 2006; Nostro et al., 2008; Murry and Keller, 2008). The majority of *in vitro* chondrogenic differentiation strategies rely on the establishment of dense pellet or micromass cultures in serum-containing or conditioned media to mimic mesenchymal condensation. However, the 3D clustering of heterogeneous cell populations creates an unknown culture environment that obscures the effects of exogenous factors due to the increased occurrence of fate-determining paracrine interactions among various cell populations, in comparison with 2D cultures, while the presence of serum components masks the effects of growth factors.

Here, we developed a monolayer SF ESC differentiation culture method to facilitate imaging and study the effects of mesoderm inducers on the generation of ESC-derived mesodermal progenitors and chondrocytes. Short-term exposure of ESCs to activin A or BMP4 was sufficient to induce chondrocyte formation, while Wnt3a only promoted chondrogenic differentiation as a late inducer after mesoderm specification.

Results

N2B27-based SF medium supported ESC adhesion and proliferation on collagen IV

We examined the combined effects of LIF-supplemented SF medium formulations, ECMs, and seeding densities on OCT4 expression in undifferentiated ESCs. LIF- and BMP4-supplemented N2B27 medium has been previously shown to maintain undifferentiated ESCs in culture (Ying et al., 2003a). N2B27 has also been used in various ESC mesoderm differentiation studies (Gadue et al., 2006; Nostro et al., 2008; Purpura et al., 2008). Chemically defined medium (CDM) was formulated to study the roles of activin A and BMP4 in mesoderm and hematopoietic development (Johansson and Wiles, 1995); while X-Vivo™10 (X-Vivo) was developed for human hematopoietic cells and ESC cultures. For ECM selection, gelatin has been widely used in ESC cultures, gelatin + fibronectin has been commonly used in high content imaging (HCI) assays (Davey and Zandstra, 2006; Walker et al., 2007) and collagen IV has been used in serum monolayer differentiation cultures (Fujiwara et al., 2007; Nishikawa et al., 1998; Tada et al., 2005; Sakurai et al., 2006). Seeding densities were set at 60, 000; 30, 000; and 15, 000 cells/cm^2.

Histograms representing OCT4 expression profiles were generated from the fluorescence intensity data quantified by HCI analysis (Supplementary Methods). Regardless of seeding density or medium formulation, 2-day cultures established on gelatin + fibronectin demonstrated significant loss in OCT4 expression with a clear separation between OCT4$^+$ and OCT4$^-$ populations, while OCT4 levels remained high in gelatin or collagen IV cultures (Figs. 1A and B, Supplementary Fig. 1A). OCT4 expression decreased in gelatin + fibronectin cultures as cell density decreased (Fig. 1C, i) while it varied in collagen IV cultures depending on SF medium formulation and remained steady in gelatin cultures (Supplementary Fig. 1B). OCT4 levels decreased and varied greatly in collagen IV cultures seeded at 15, 000 cells/cm^2 in X-Vivo cultures due to the scarcity of colonies, while the opposite trend was observed in CDM cultures caused by overconfluency (Fig. 1C, ii). Therefore, ESC cultures established on collagen IV and maintained in LIF-supplemented N2B27-based SF medium were most tolerant to varying seeding densities.

Activin A-supplemented monolayer differentiation cultures exhibited stronger cell-matrix adhesion and improved survival

We investigated the roles of activin A (30 ng/ml), BMP4 (10 ng/ml), and Wnt3a (100 ng/ml) in our SF monolayer culture system as they effectively induced mesoderm formation in EB studies (Murry and Keller, 2008). Activin A acts as a surrogate of Nodal by signaling through the same receptor but is not inhibited by Lefty1, which is expressed during EB development (Gadue et al., 2006). Morphological examination of 4-day differentiating ESCs under serum conditions showed extensive cell spreading and strong cell-matrix adhesion (Fig. 2A, i). Differentiating cells in activin A- or Wnt3a-supplemented cultures adhered well on collagen IV-coated surface (Fig. 2A, ii and iii), albeit with inferior cell spreading and distribution compared to serum cultures. Some colonies in BMP4 cultures elevated from the flattened surrounding cells and displayed poor cell-matrix contact (Fig. 2A, iv). Overall, growth factor-supplemented cultures displayed stronger cell-matrix adhesion than untreated SF cultures consisting of tightly packed colonies that detached easily from the culture surface (Fig. 2A, v).

We determined whether differences in cell adhesion capabilities in growth factor-supplemented cultures were indicative of a compromise in cell survival, specifically that of the nascent mesodermal cells. Flow cytometric analysis (Supplementary Methods) of Flk1:eGFP ESCs (Ema et al.,

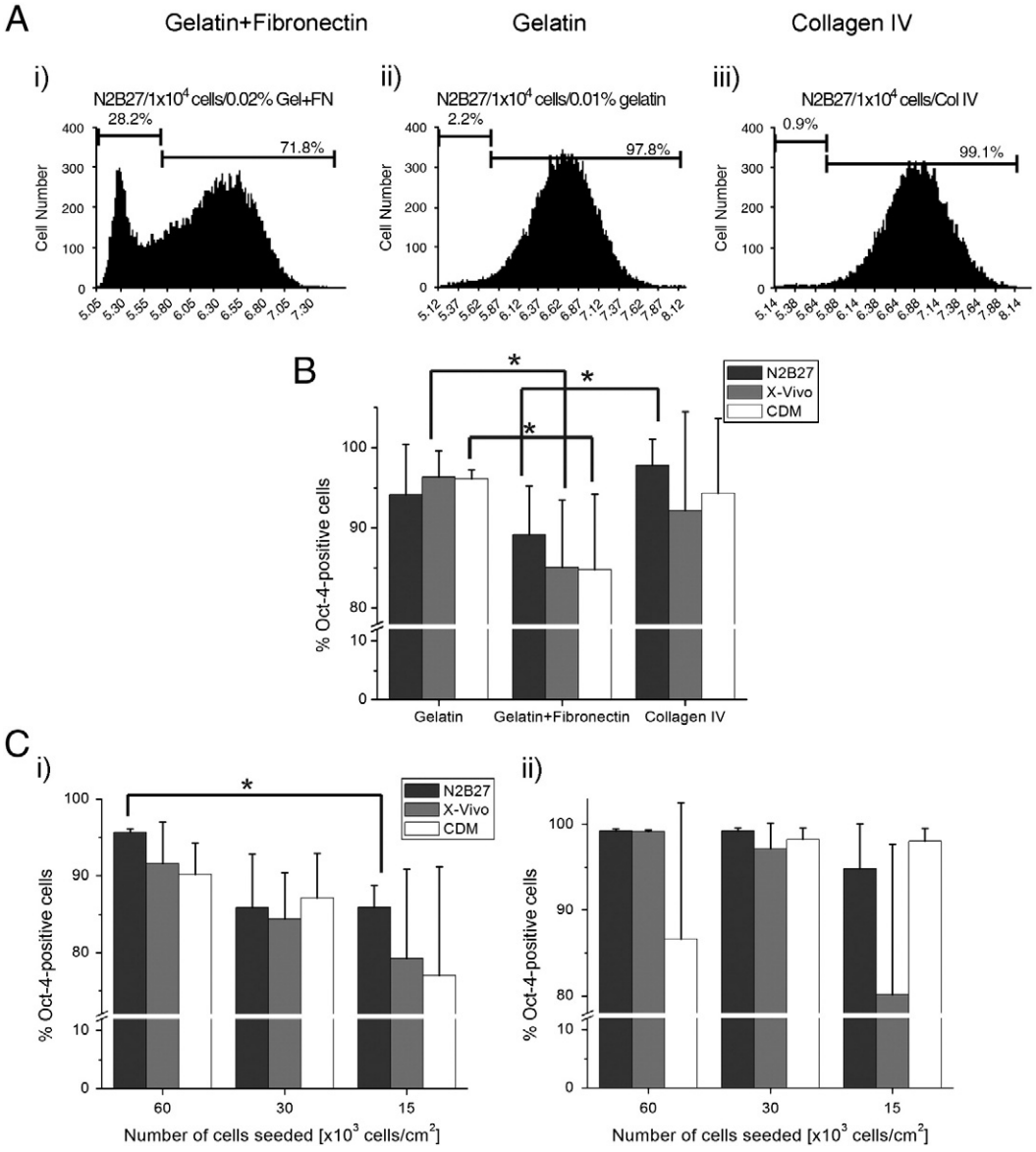


Figure 1 Two-day ESC cultures on collagen IV in N2B27-based SF medium with LIF maintained high OCT4 expression. (A) N2B27 cultures on (i) gelatin + fibronectin exhibited a biomodal distribution of OCT4 levels compared to those on (ii) gelatin and (iii) collagen IV, leading to a lower percentage of $OCT4^+$ population (B). (C) (i) OCT4 levels of gelatin + fibronectin cultures reduced with decreasing seeding density regardless of SF medium formulation. (ii) OCT4 expression varied dramatically at a high seeding density in X-Vivo cultures on collagen IV, while the opposite trend was observed in CDM cultures. OCT4 expression remained relatively stable at all seeding densities in N2B27 cultures on collagen IV. Plotted values represented means ± SEM ($n = 2$).

2006) differentiated for 4 days in SF monolayer cultures showed that Flk1:eGFP expression was significantly higher in activin A-supplemented cultures compared to untreated cultures (Fig. 2B). Interestingly, greater *Flk1* transcript expression was observed in BMP4- or Wnt3a-supplemented cultures than that in activin A-treated cultures (Fig. 2C). Expression of the apoptotic marker annexin V was significantly higher in BMP4-supplemented cultures than in activin A-treated cultures (Fig. 2D). Dot plots showed that <1% of the population coexpressed Flk1:eGFP and annexin V (Fig. 2E), indicating that monolayer ESC cultures established on collagen IV in growth factor-supplemented N2B27-based SF medium supported mesoderm differentiation.

Endogenous *Wnt3a* was up-regulated in serum cultures as well as BMP4-supplemented and untreated SF differentiation cultures

Endogenous *BMP4*, *Nodal*, and *Wnt3a* expression in SF differentiation cultures was quantified by qPCR to determine if they were specifically up-regulated by their respective exogenous ligands. Activin A-supplemented cultures had significantly higher *Nodal* transcript levels than BMP4-supplemented and untreated 4-day SF cultures (Fig. 2F). Interestingly, *BMP4* expression was up-regulated by either BMP4 or Wnt3a ligand (Fig. 2G). The expression of *Nodal*, *BMP4*, and *Wnt3a* increased competitively in serum cultures. Surprisingly, *Wnt3a* expression

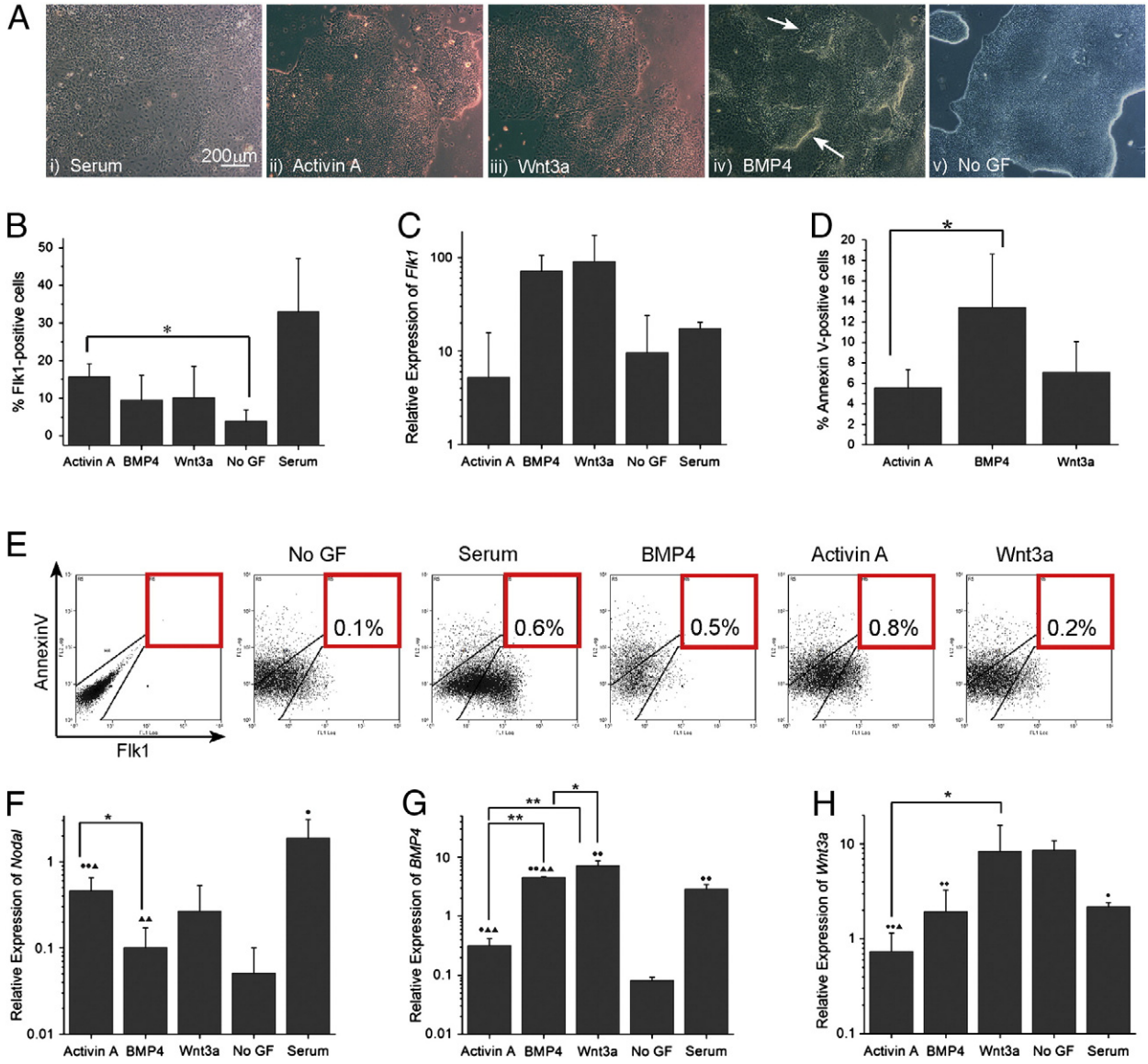


Figure 2 Characteristics of 4-day ESC SF monolayer differentiation cultures established on collagen IV. (A) Images (100×) taken with a camera-mounted Leica DM IL inverted microscope illustrated that serum cultures (i) exhibited more pronounced cell adhesion and spreading than SF activin A (ii) and Wnt3a (iii) cultures. Raised colonies (arrows) were present in BMP4 cultures (iv), while untreated cultures ("No GF") (v) consisted of tightly packed cell populations that adhered poorly. (B) Flow cytometry analyses and (C) qPCR indicated that although activin A, BMP4, and Wnt3a induced Flk1:eGFP expression and *Flk1* transcript in SF monolayer differentiation cultures, respectively, expression of the apoptotic marker annexin V was distinctly higher in BMP4 cultures (D). (E) Flow cytometric dot plots of annexin V vs Flk1:eGFP showed that nascent mesodermal cells generated in the SF monolayer cultures were not apoptotic, as <1% of the population expressed both markers (highlighted in red). In examining potential synergistic effects of activin A, BMP4, and Wnt3a, qPCR analyses of endogenous expression of (F) *Nodal*, (G) *BMP4*, and (H) *Wnt3a* mRNA showed that *Wnt3a* was significantly up-regulated in BMP4-supplemented cultures. Plotted values from flow cytometry analyses and qPCR represent means ± SEM ($n \geq 2$).

was markedly up-regulated to comparable levels in both untreated and Wnt3a-supplemented cultures (Fig. 2H).

BMP4, activin A, or Wnt3a induced Brachyury-positive primitive streak-like populations in monolayer differentiation cultures

To corroborate with the Flk1 results (Fig. 2B), 4-day monolayer cultures were immunostained (Supplementary Methods) for expression of the primitive streak/early mesoderm marker Brachyury. Activin A-, Wnt3a-, or serum-supplemented cultures showed comparable levels of Brachyury expression (Fig. 3A, i–iii). BMP4 did not induce Brachyury expression as robustly as activin A or Wnt3a (Fig. 3A, iv); however, qPCR analysis measured similar *Brachyury* transcript levels in all growth factor-supplemented cultures (Fig. 3B). HCI and immunofluorescence (IF) analyses showed that Brachyury was induced in a dose-dependent manner (Fig. 3C, Supplementary Fig. 1C). Activin A or Wnt3a addition

to BMP4-supplemented cultures increased Brachyury expression, suggesting that activin A and Wnt3a were synergistic inducers of primitive streak-like cells at the tested concentrations (Supplementary Fig. 1D, i and ii) but their simultaneous presence did not further increase Brachyury expression (Supplementary Fig. 1D, iii). Untreated SF medium alone did not induce noticeable Brachyury expression (Fig. 3A, v). As expected, Brachyury expression was significantly higher in serum-containing cultures than under SF conditions, while serum components masked the inductive effect of activin A on Brachyury expression (Fig. 3D, Supplementary Fig. 1D, iii).

Mesoderm marker genes expression patterns correlated with those in EB cultures and in murine embryos studies

Previous reports showed that BMP4 has a posteriorizing effect on differentiating ESCs, while activin A promotes the formation of increasingly more anterior populations in a concentration-dependent manner (Murry and Keller, 2008). Based on marker expression, we observed similar growth factor-dependent enrichment of mesoderm subsets in our 4-day monolayer cultures. Expression of the posterior mesoderm markers *Evx1*, *HoxB1*, *Tal1*, and *GATA2* was dramatically up-regulated in BMP4-supplemented cultures (Fig. 3E, i–iv). *Lhx1* expression was induced by activin A, BMP4, and Wnt3a (Fig. 3E, v), while BMP4 and Wnt3a supplementation led to higher transcript levels of the paraxial mesoderm marker *PDGFRα* compared to activin A (Fig. 3E, vi). Activin A and Wnt3a, but not BMP4, induced the expression of the anterior marker *Meox2* (Fig. 3E, vii), while all three growth factors exerted similar inductive effects on *Fst* and *Mesp2* expression (Fig. 3E, viii and ix). As expected, expression of the mesendoderm markers *Gsc* and *Foxa2* was up-regulated by activin A (Fig. 3E, x and xi). Wnt3a appeared to have a pan-mesodermal inductive effect based on transcript level analysis. Therefore, differential growth factor supplementation at the onset of differentiation facilitated enrichment of mesoderm subsets in monolayer cultures without cell sorting.

Activin A facilitated chondrogenic differentiation in SF monolayer cultures

Fifteen-day growth factor-supplemented monolayer cultures were established to determine if prolonged exposure to mesoderm inducers could trigger chondrogenic induction. Activin A-supplemented (30 ng/ml) cultures (Fig. 4A, i) showed more intense Alcian blue staining than those treated with BMP4 (10 ng/ml), Wnt3a (100 ng/ml), or serum (Fig. 4A, ii–iv); also, robust Col2a1 networks were only present in activin A-supplemented cultures (Fig. 4B). Sox9-positive cells were also present in activin A-supplemented cultures (Fig. 4C). Similar to 4-day cultures, 15-day untreated SF differentiation cultures demonstrated poor cell-matrix adhesion with the formation of EB-like structures that were loosely anchored via filamentous protrusions (Fig. 4D) and were easily dislodged during media replenishment. Real-time qPCR analysis showed that activin A-supplemented cultures showed marked up-regulation of the chondrogenic markers *Col2a1, Sox9, and Aggrecan* while cultures with BMP4, Wnt3a and serum showed minimal changes in gene expression (Figs. 4E–G). Activin A did not strongly enhance the expression of *Col10a* and *Runx2* compared to noninductive conditions (Fig. 4H), suggesting the maintenance of nonhypertrophic chondrocytes after 15 days of differentiation.

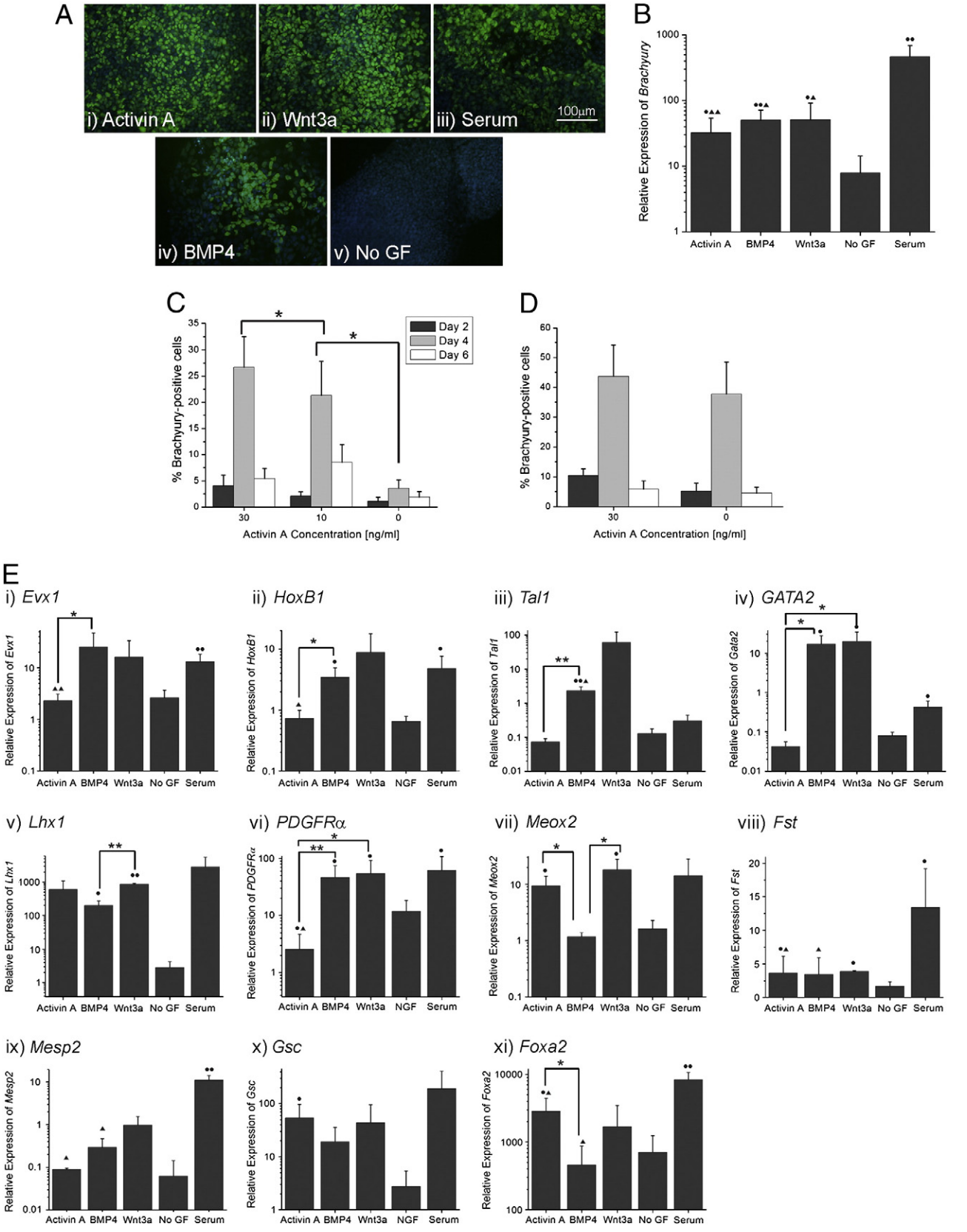
Comparison of the expression levels of chondrogenic marker genes between monolayer and micromass cultures suggested that both systems behaved similarly. Interestingly, we were unable to generate micromasses in cultures supplemented with chondrogenic media (Woods et al., 2007) due to poor adhesive properties of the droplets. Micromass cultures established in N2B27-based SF medium supplemented with activin A (30 ng/ml) exhibited similar levels of *Col2a1* and *Col10a* as our monolayer cultures, with slightly enhanced *Sox9* levels and lower *Aggrecan* expression (Supplementary Fig. 2A compared to Figs. 4E–H). However, IF analysis of Col2a1 expression highlighted the shortcoming of the 3D micromass culture in that clear network formation could only be visualized at the periphery of the micromass (Supplementary Fig. 2B compared to Fig. 4B, i), while the resolution of image deteriorated toward the center of the micromass. As such, it would be difficult to quantify protein expression in these 3D cultures using HCI strategies.

TGFβ3 induced chondrocyte formation when added at the onset of differentiation

Treatment of SF monolayer cultures with TGFβ3 (10 ng/ml) (Fig. 4I, i and ii) or FGF8 (50 ng/ml) (Fig. 4I, iii and iv) induced chondrogenic differentiation with evident Col2a1 network formation beginning on Day 7 of differentiation. However, cells cultured in FGF8 alone exhibited poor cell-matrix adhesion similar to untreated SF cultures. Aside from Col2a1, both FGF8- and TGFβ3-treated cultures also possessed Sox9-expressing populations (Fig. 4J). TGFβ3 and FGF8 were not superior to activin A in their chondrogenic

Figure 3 Early mesoderm specification in 4-day growth factor-supplemented SF monolayer differentiation cultures. (A) IF images (200×) showed that (i) activin A, (ii) Wnt3a, and (iii) serum cultures displayed comparable Brachyury protein levels, while (iv) BMP4 cultures appeared to have less Brachyury^{+} cells. (v) Brachyury was not detected in untreated cultures. However, (B) qPCR results suggested that activin A, BMP4, and Wnt3a exerted similar inductive effects on *Brachyury* transcription while serum effect was the most potent. Plotted values represent means ± SEM ($n \geq 3$). (C) HCI analysis demonstrated that Brachyury protein level was directly correlated with growth factor concentration (e.g., activin A), while this effect was masked in serum cultures (D). Plotted values represent means ± SEM ($n = 2$). (E) qPCR analysis indicated that BMP4 was more inductive in the up-regulation of the posterior primitive streak markers (i) *Evx1*, (ii) *HoxB1*, (iii) *Tal1*, and (iv) *GATA2*, while activin A appeared to be equally inductive in (v) *Lhx1*. (vi) BMP4 was more effective in inducing the paraxial mesoderm marker *PDGFRα* than activin A, which up-regulated the anterior mesoderm marker (vii) *Meox2*, (viii) *Fst*, and (ix) *Mesp2* to a lesser extent. Activin A effectively up-regulated the mesendoderm markers (x) *Gsc* and (xi) *Foxa2*, and Wnt3a appeared to have a pan-mesodermal inductive effect. Plotted values represent means ± SEM ($n \geq 2$).

inductive abilities; *Sox9* and *Col10a* transcript expression only increased minimally in TGFβ3- or FGF8-supplemented cultures compared to activin A-supplemented cultures (Fig. 4K). Col2a1 protein was undetectable in BMP4-supplemented cultures treated with TGFβ3 or FGF8 beginning on Day 5 of differentiation (Fig. 4L, i and ii). Despite its



confirmed role as a chondrogenic inducer, activin A addition to BMP4-supplemented cultures failed to induce Col2a1 or proteoglycans production (Supplementary Figs. 3A and B). These data suggest that either BMP4 exerted a dominant chondrogenic inhibitory effect on our SF monolayer cultures, or activin A, TGFβ3, and FGF8 functioned early on during chondrogenic induction. Although strong Col2a1 networks were formed in activin A-supplemented cultures containing TGFβ3 or FGF8 (Fig. 4L, iii and iv) and this result was corroborated by Alcian blue staining (Fig. 4M), qPCR results indicate that TGFβ3 or FGF8 addition to activin A-supplemented cultures did not further enhance chondrogenic markers gene expression. Interestingly, the presence of TGFβ3 or FGF8 in BMP4-supplemented cultures increased the gene expression of *Col2a1*, *Sox9*, and *Aggrecan* (in the case of TGFβ3) compared to BMP4 alone (Fig. 4N).

Five-day activin A treatment achieved competitive chondrogenic differentiation in SF monolayer cultures

Since our data suggest that activin A acts as an early inducer of chondrogenic differentiation, we examined the feasibility of shortening the duration of supplementation. Robust Col2a1 networks were present in 15-day SF monolayer cultures supplemented with activin A for the first 5 days of differentiation but not in BMP4 or Wnt3a cultures (Fig. 5A, i–iii). Similarly, Sox9 protein expression was detected in activin A-supplemented cultures (Fig. 5B, i); however, there appeared to be very weak Sox9 expression in Wnt3a-treated cultures also (Fig. 5B, iii). In terms of transcript levels, 5-day activin A supplementation led to increased expression of *Col2a1*, *Sox9*, *Aggrecan*, and *Col10a* compared to BMP4- and Wnt3a-treated cultures (Fig. 5C).

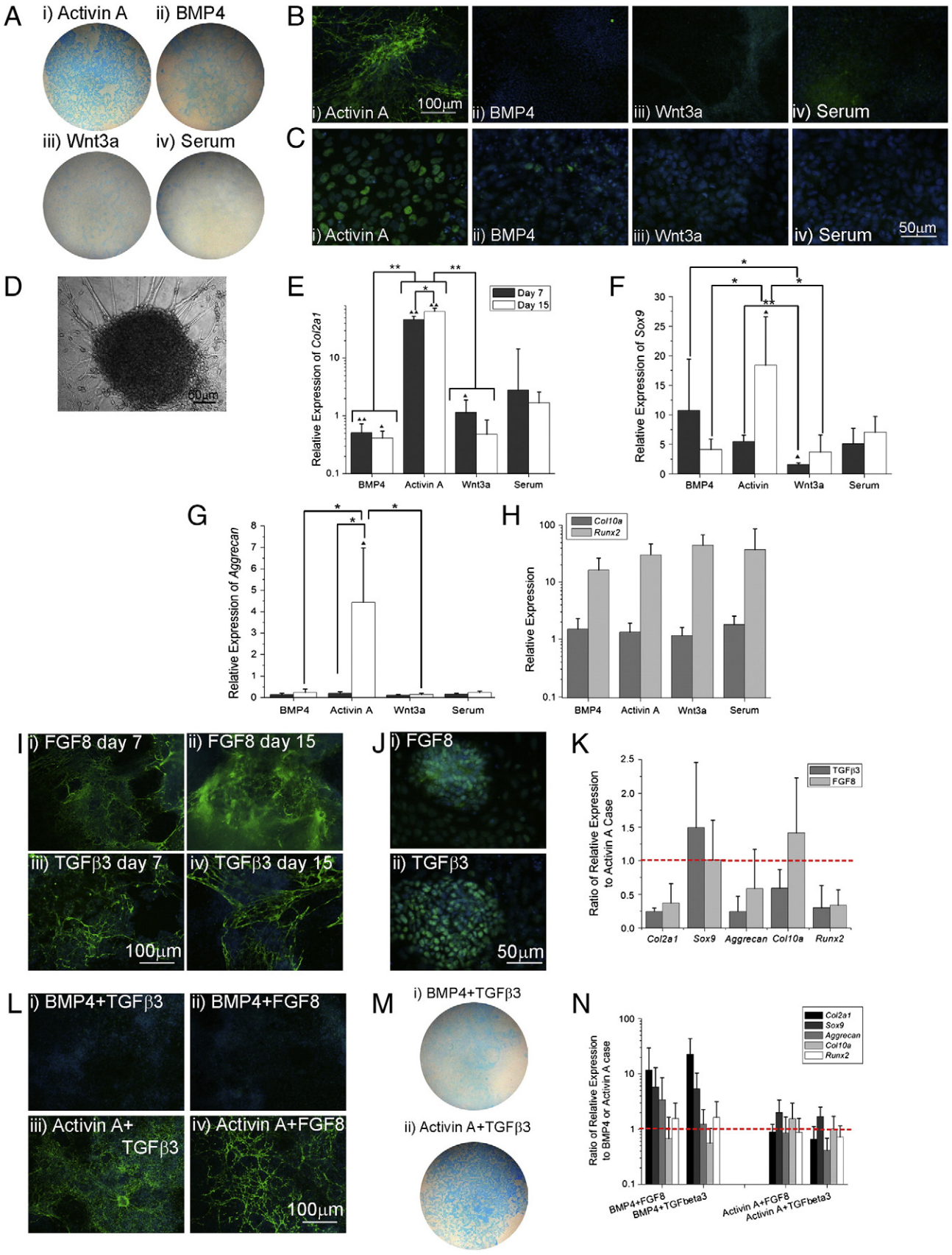
We examined whether the noninductive BMP4 (10 ng/ml) and Wnt3a (100 ng/ml) would inhibit the progression of chondrogenic induction initiated by 5-day activin A supplementation. IF data indicated that BMP4 or Wnt3a addition from Days 5–15 of differentiation to activin A-supplemented cultures did not hinder Col2a1 network formation (Fig. 5D, i and ii). The replacement of BMP4 or Wnt3a with TGFβ3 or FGF8 did not further enhance Co2a1 protein expression in differentiation cultures initiated by activin A (Fig. 5D, iii and iv). However, the presence of FGF8 or TGFβ3 in activin A-supplemented cultures increased *Aggrecan* expression, while those with TGFβ3 showed up-regulation in *Sox9* and *Runx2* to a very small extent. FGF8 treatment also led to a marginal increase in *Col10a* expression. Interestingly, addition of Wnt3a to activin A-supplemented differentiation cultures also marginally enhanced *Sox9*, *Aggrecan*, and *Runx2* expression, suggesting that Wnt3a may play a minor role as a chondrogenic inducer (Fig. 5E). Similar to the results obtained from cultures with continuous BMP4 supplementation, activin A, TGFβ3, or FGF8 addition to cultures with 5-day BMP4 treatment did not induce Col2a1 expression (Supplementary Fig. 3C, i–iii).

Fifteen-day cultures with continuous activin A supplementation maintained higher expression of the articular cartilage-specific *PRG4* than those subjected to a 5-day treatment regime (Fig. 5F). Replacement of activin A with TGFβ3 on Day 5 of differentiation induced similar or higher *PRG4* transcript levels than activin A alone, while the presence of FGF8 did not further promote *PRG4* expression regardless of the length of activin A supplementation. Therefore, sustained activin A supplementation or the combination of activin A and TGFβ3 appeared to facilitate articular chondrocyte formation in our SF monolayer culture system.

High BMP4 concentration induced chondrogenic differentiation, while Wnt3a acted as a late chondrogenic inducer

To further investigate the role of BMP4 and Wnt3a in our SF monolayer chondrogenic cultures, we differentiated ESCs in the presence of Wnt3a (100 ng/ml) (for 15 days or 5 days) followed by BMP4 (10 ng/ml) (added from Days 5–15 of culture) and in cultures supplemented by the two growth factors in the reversed order. IF analyses of Col2a1 deposition confirmed that Wnt3a was ineffective in chondrogenic induction when added on Day 0 of differentiation, while further addition of BMP4 exerted minimal effect on chondrogenic differentiation (Fig. 6A, i and ii). Addition of activin A, TGFβ3, or FGF8 to

Figure 4 Activin A- or TGFβ3-supplemented SF monolayer differentiation cultures underwent chondrogenic differentiation within 15 days. (A) Fifteen-day cultures supplemented with activin A (i) were more intensely stained with Alcian blue than BMP4 (ii), Wnt3a (iiii), and serum (iv) cultures. (B) IF analyses (200×) showed that (i) activin A supplementation led to the formation of Col2a1 networks that were absent in (ii) BMP4-, (iii) Wnt3a-, and (iv) serum-supplemented cultures. (C) IF analyses (400×) also showed Sox9 expression in 11-day (i) activin A-supplemented cultures but not in those treated with (ii) BMP4, (iii) Wnt3a, or (iv) serum. (D) Image (400×) taken using a camera-mounted Leica DM IL inverted microscope showed that untreated SF cultures adhered poorly and formed aggregates that were loosely anchored on the culture surface via filamentous protrusions. Real-time qPCR results confirmed the up-regulation of (E) *Col2a1*, (F) *Sox9*, and (G) *Aggrecan* in Day 7 and Day 15 of activin A-supplemented SF monolayer differentiation cultures, while the levels of hypertrophic markers *Col10a* and *Runx2* were similar to those under noninductive conditions (H). Plotted values in E–H represent means ± SEM ($n \geq 3$). (I) FGF8 (i, ii) and TGFβ3 (iii, iv) were also found to induce Col2a1 expression when added alone to SF monolayer differentiation cultures. However, FGF8 cultures exhibited similar morphology as (D). (J) Eleven-day (i) FGF8- and (ii) TGFβ3-treated cultures also consisted of Sox9-positive populations. (K) Compared to activin A, TGFβ3 appeared to be more potent in inducing *Sox9* expression, while FGF8 further up-regulated *Col10a* transcript level. (L) When added as a potential enhancer to BMP4 (i, ii) or activin A (iii, iv), TGFβ3 and FGF8 did not have a noticeable effect on Col2a1 expression, as supported by Alcian blue staining (M). Interestingly, the addition of those two factors to BMP4-supplemented cultures markedly improved *Col2a1*, *Sox9*, and *Aggrecan* expression from BMP4 addition alone. However, their effects were not as pronounced in activin A-supplemented cultures (N). Plotted values in K and N represent ratios of means ± relative errors ($n \geq 3$).


A
i) Activin A
ii) BMP4
iii) Wnt3a
iv) Serum
B
i) Activin A
100μm
ii) BMP4
iii) Wnt3a
iv) Serum
C
i) Activin A
ii) BMP4
iii) Wnt3a
iv) Serum
50μm
D
50μm
E
Relative Expression of Col2a1
Day 7
Day 15
BMP4
Activin A
Wnt3a
Serum
F
Relative Expression of Sox9
BMP4
Activin
Wnt3a
Serum
G
Relative Expression of Aggrecan
BMP4
Activin A
Wnt3a
Serum
H
Col10a
Runx2
Relative Expression
BMP4
Activin A
Wnt3a
Serum
I
i) FGF8 day 7
ii) FGF8 day 15
iii) TGFβ3 day 7
iv) TGFβ3 day 15
100μm
J
i) FGF8
ii) TGFβ3
50μm
K
TGFβ3
FGF8
Ratio of Relative Expression to Activin A Case
Col2a1
Sox9
Aggrecan
Col10a
Runx2
L
i) BMP4+TGFβ3
ii) BMP4+FGF8
iii) Activin A+ TGFβ3
iv) Activin A+FGF8
100μm
M
i) BMP4+TGFβ3
ii) Activin A+TGFβ3
N
Col2a1
Sox9
Aggrecan
Col10a
Runx2
Ratio of Relative Expression to BMP4 or Activin A case
BMP4+FGF8
BMP4+TGFbeta3
Activin A+FGF8
Activin A+TGFbeta3

Wnt3a-supplemented cultures did not facilitate Col2a1 network formation or proteoglycans production (Supplementary Figs. 3D and E), reinforcing their described roles as early chondrogenic inducers. When the order of Wnt3a and BMP4 administration was reversed, however, Col2a1 networks were successfully formed (Fig. 6A, iii and iv). Compared to cultures supplemented with Wnt3a at the onset of differentiation, treatment with BMP4 followed by Wnt3a led to enhanced expression of *Col2a1* and *Sox9* as well as the late chondrogenic marker *Col10a* and the osteogenic transcription factor *Runx2* (Fig. 6B), suggesting that Wnt3a acted as a late inducer of chondrogenesis in place of BMP4 (10 ng/ml). Alcian blue staining was only present in cultures supplemented with BMP4 followed by Wnt3a (Fig. 6C, iii and iv) albeit with less intensity than those present in chondrogenic cultures induced by activin A. The decreased proteoglycan deposition was also reflected in *Aggrecan* transcript expression levels (Fig. 6B).

Contrary to published data establishing BMP4 as an inducer of ESC chondrogenesis (Vinatier et al., 2009; van Osch et al., 2009; Heng et al., 2004), BMP4 (10 ng/ml) does not have an appreciable effect on chondrogenic differentiation in our culture system. To explain this disparity, we examined the concentration effect of BMP4 on our monolayer differentiation cultures. With 25 ng/ml of BMP4, robust Col2a1 networks were evident regardless of the duration of supplementation (Fig. 6D, i and ii). Similar to activin A, addition of TGFβ3 or FGF8 to BMP4-supplemented cultures showed positive Col2a1 staining as BMP4 alone (Fig. 6D, iii and iv, v and vi, respectively). IF data were corroborated by qPCR, although overall transcript levels were lower than those in activin A-treated cultures (Fig. 6E). Specifically, *Aggrecan* gene expression was comparable to data obtained from cultures treated with BMP4 (10 ng/ml) followed by Wnt3a and was similarly reflected by Alcian blue staining (Fig. 6F). These findings confirm that activin A (30 ng/ml) and TGFβ3 (10 ng/ml) are more effective than BMP4 (25 ng/ml) as early inducers of chondrogenesis.

Discussion

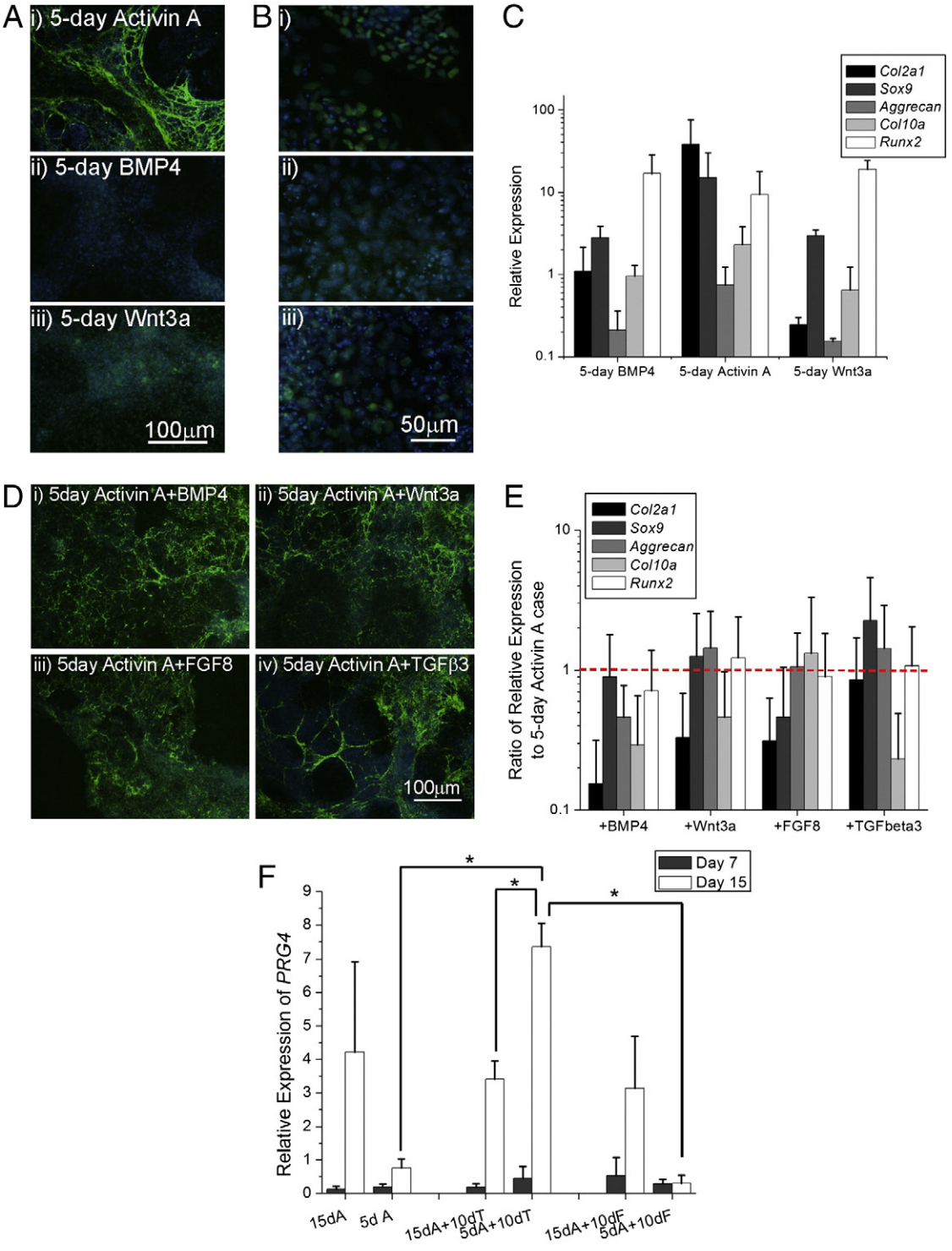
We examined if long-term treatment of murine ESC SF monolayer cultures with BMP4, activin A, Wnt3a, TGFβ3, and FGF8 at the onset of differentiation could direct chondrogenic differentiation with minimal culture manipulation. Activin A, BMP4, and TGFβ3 acted as early inducers of chondrogenesis in our culture system while Wnt3a exerted its prochondrogenic effect only after mesoderm specification (Fig. 7).

ECM selection was crucial as 2-day LIF-supplemented SF ESC cultures established on gelatin + fibronectin exhibited accelerated differentiation with 5–10% lower OCT4 levels compared to cultures on gelatin or collagen IV (Figs. 1A and B). The bimodal OCT4 expression profile was commonly observed in other HCI assays established on gelatin + fibronectin (Davey and Zandstra, 2006; Walker et al., 2007). Although fibronectin is endogenously expressed by differentiating ESCs (Hayashi et al., 2007) and promotes cell adhesion and spreading (Dufour et al., 1986), we rejected gelatin + fibronectin for our system to minimize spontaneous ESC differentiation toward undesired lineages in the absence of inductive factors. Gelatin, being a mixture of collagens, was less defined than collagen IV, and collagen IV has been shown to facilitate ESC differentiation toward the mesodermal lineages (Nishikawa et al., 1998; Tada et al., 2005; Sakurai et al., 2006). Despite the successful establishment of adherent chondrocyte cultures on fibronectin or collagen (Khan et al., 2009; Ho et al., 2009), gelatin + fibronectin cultures did not show enhanced Brachyury or chondrogenic markers expression compared to collagen IV cultures (data not shown).

Similar to published EB studies (Nostro et al., 2008), BMP4, activin A, and Wnt3a all induced Flk1 (Figs. 2B and C) and Brachyury expression (Figs. 3A and B) in our 4-day monolayer differentiation cultures. Activin A- or Wnt3a-treated cultures consisted of flattened colonies with stronger adhesion and spreading on collagen IV than with BMP4 (Fig. 2A). Conversely, there was ~6–8% more of annexin V^+ apoptotic cells present in BMP4 cultures than in Wnt3a and activin A cultures (Fig. 2C). Activation of the TGFβ signaling pathway induces epithelial-to-mesenchymal transition (EMT), leading to the up-regulation of neural cell adhesion molecule (NCAM) (Thiery and Sleeman, 2006). NCAM promotes the phosphorylation of focal adhesion kinase (FAK) and integrin-dependent cell spreading (Frame and Inman, 2008). FAK phosphorylation alters its downstream target Grb2 and facilitates its interaction with the Ras/mitogen-activated protein kinase (MAPK) pathway, which modulates cell survival and proliferation (Schlaepfer et al., 1994; Harburger and Calderwood, 2009). Members of the

Figure 5 Chondrogenic differentiation was achieved in SF monolayer cultures supplemented with activin A from Day 0 to Day 5 of differentiation. (A) IF images (200×) showed that (i) activin A addition on the first 5 days of differentiation was sufficient to generate Col2a1 networks in 15-day cultures, while (ii) BMP4 and (iii) Wnt3a treatment failed to do so. (B) IF analyses (400×) also confirmed the expression of Sox9 in 11-day cultures subjected to 5-day activin A supplementation while (ii) BMP4- and (iii) Wnt3a had faint to no positive staining. (C) Real-time qPCR analyses of 5-day supplementation cultures reflected similar up-regulation patterns in *Col2a1, Sox9* and *Aggrecan* as 15-day supplementation cultures. *Col10a* transcript level was slightly increased in cultures with 5-day activin A treatment, but *Runx2* expression was unaffected. Plotted values represent means ± SEM ($n \geq 3$). In examining if sequential addition of growth factors led to enhanced chondrogenic induction, (D) IF images (200×) of Day 15 differentiation cultures supplemented with activin A for the first 5 days followed by 10-day addition of (i) BMP4, (ii) Wnt3a, (iii) FGF8, and (iv) TGFβ3 all showed a similar extent of Col2a1 network formation. (E) Real-time qPCR results suggested that culture supplemented with activin A for 5 days followed by BMP4 for 10 days did not have a notable effect on chondrogenic induction. Replacement of BMP4 with FGF8 caused a marginal increase in *Aggrecan* and *Col10a* expression, while addition of Wnt3a or TGFβ3 facilitated slight increases in *Sox9*, *Aggrecan*, and *Runx2*. Plotted values represent ratios of means ± relative errors ($n \geq 3$). (F) Real-time qPCR analysis of *PRG4* expression indicated that sustained activin A supplementation ("A") or the sequential addition of activin A followed by TGFβ3 ("T") appeared to promote articular chondrocyte formation. Plotted values represent means ± SEM ($n \geq 3$). Note: "F" = FGF8.

canonical Wnt and integrin signaling pathways (specifically the collagen-binding integrins $\alpha1\beta1$ and $\alpha2\beta1$) have been shown to act synergistically via Grb2, (Crampton et al., 2009), possibly contributing to the satisfactory cell spreading and survival observed in Wnt3a-supplemented cultures. The non-uniform morphology of differentiating colonies in BMP4 culture (Fig. 2A, iv) could be due to the potency of BMP4 at 10 ng/ml as the cultures also showed weaker Brachyury protein expression (Fig. 3A, iv); consequently, serum-deprivation promoted apoptosis in the slowly differentiating cells. Alternatively, although BMP4 (10 ng/ml) was less potent than activin A or Wnt3a in EMT initiation and mesoderm induction, its presence was sufficient to prevent neuroectoderm differentiation in our culture system by inducing apoptosis in early precursors of neural cells (Gambaro et al., 2006).



Up-regulation of endogenous *Wnt3a* in both 4-day untreated and BMP4-supplemented monolayer differentiation cultures (Fig. 2H) suggested possible cross talk between BMP4 and Wnt3a. Recently, exogenous BMP4 was found to cause increases in *Wnt3a* levels in a similar monolayer differentiation system and both signaling pathways functioned synergistically to induce different mesoderm populations (Tanaka et al., 2009). Components in the N2B27 medium may also induce *Wnt3a* expression that contributed to the seemingly pan-mesodermal inductive ability of the Wnt3a ligand (Fig. 3E). However, qPCR data from 15-day cultures showed that exogenous Wnt3a was less inductive than activin A in the expression of the anterior cardiac markers *αMHC* and *Nkx2.5* (Supplementary Fig. 4A, i and ii) while it promoted the expression of the posterior hematopoietic marker *GATA1* (Supplementary Fig. 4A, iii). These data were consistent with reports showing that Wnt3a inhibited cardiomyocyte differentiation after mesoderm induction (Naito et al., 2006; Ueno et al., 2007). Our SF monolayer differentiation system did not appear to support the formation of definitive endoderm (Supplementary Fig. 4A, iv).

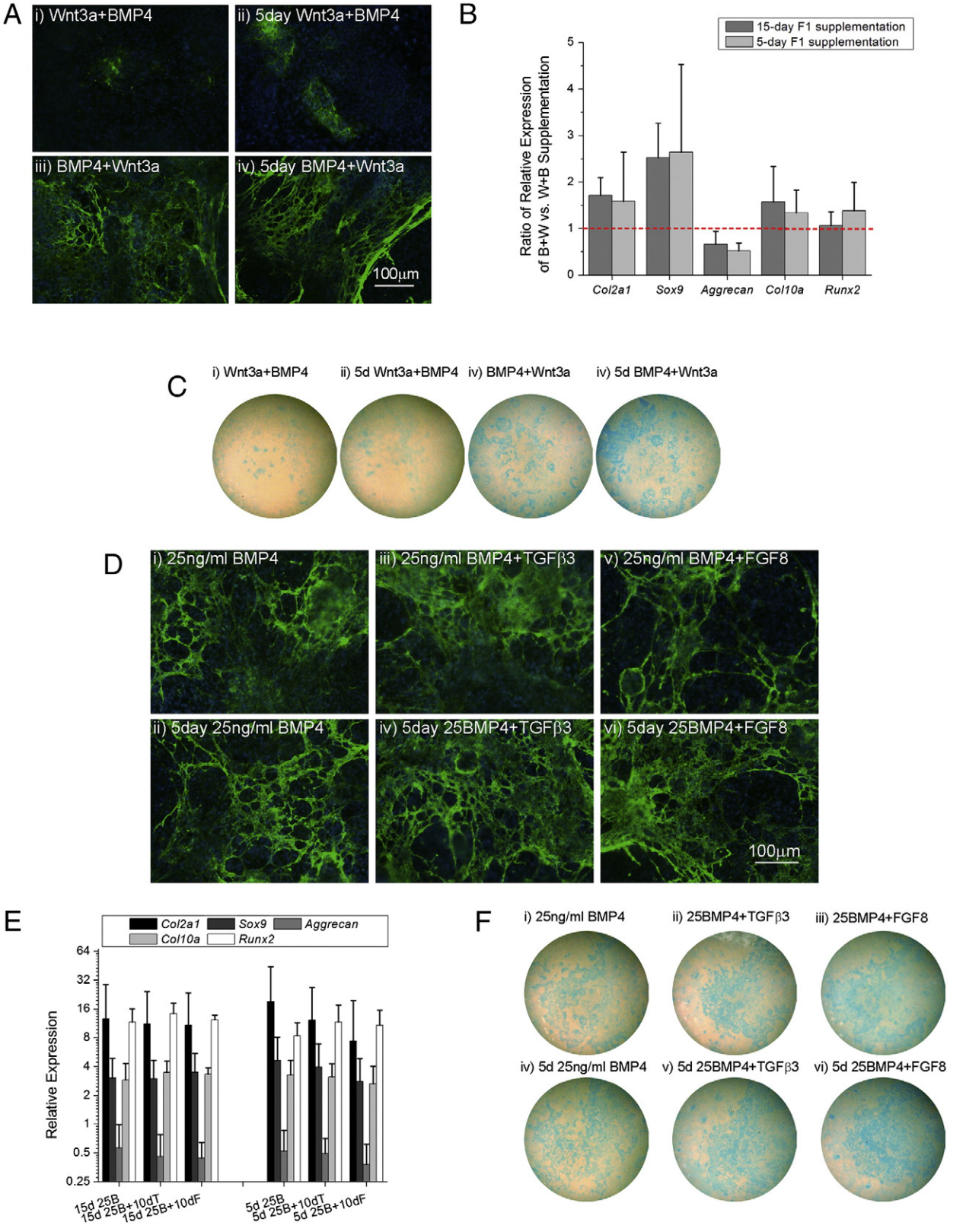
Posterior and anterior mesodermal populations were enriched without cell sorting in growth factor-supplemented SF monolayer cultures. Similar to EB studies (Gadue et al., 2006), there were clear increases in posterior mesoderm marker genes expression (*Evx1*, *HoxB1*, *Tal1*, and *GATA2*) in BMP4-supplemented cultures compared to activin A-supplemented cultures (Fig. 3E, i–iv), while the mesendoderm markers *Gsd* and *Foxa2* exhibited the opposite expression patterns (Fig. 3E, x and xi). This distinction was not as definitive in the expression of paraxial mesoderm marker genes like *Fst* and *Mesp2* (Fig. 3E, viii and ix). This phenomenon was expected because lateral plate and paraxial mesoderm populations emerge adjacent to each other in development with some overlapping gene expression patterns. Similarly, although *Lhx1* has been identified as a marker for lateral plate mesoderm (Tam and Loebel, 2007) and we anticipated higher *Lhx1* expression in BMP4-treated cultures than in activin A cultures, we observed comparable *Lhx1* up-regulation in activin A-, BMP4-, and Wnt3a-supplemented cultures (Fig. 3E, v). This was logical because *Lhx1* is a known target of the Nodal signaling pathway (Shen, 2007) and is also expressed in lateral-intermediate mesoderm, anterior mesendoderm, and visceral endoderm (Shawlot et al., 1999; Tsang et al., 2000; Tam et al., 2004). In contrast, despite the use of *PDGFRα* to characterize ESC-derived paraxial mesoderm (Sakurai et al., 2006, 2008) and reports showing that *PDGFRα* expression was induced by Nodal activation (Takenaga et al., 2007) in ESC cultures established on collagen IV (Sakurai et al., 2006), *PDGFRα* up-regulation was more responsive to exogenous BMP4 and Wnt3a than activin A in our culture system (Fig. 3E, vi). Indeed, BMP4 has also been shown to induce *PDGFRα* in ESCs (Tanaka et al., 2009; Sakurai et al., 2009) and such a reversed expression pattern has been observed in monolayer cultures of differentiating human ESCs (Lee et al., 2009).

R1 ESCs (Nagy et al., 1993) have been shown to have poor chondrogenic differentiation capabilities in EB studies (Kramer et al., 2005). Although activin A has been shown to be both an inducer (Jiang et al., 1993) and an inhibitor (Chen et al., 1993) of chondrogenic differentiation in limb bud mesodermal cells, we showed that activin A induced chondrocyte formation in our R1 SF monolayer cultures with intense Alcian blue staining (Fig. 4A), robust Col2a1 network formation (Fig. 4B), detection of Sox9 protein expression (Fig. 4C), and marked up-regulation of *Col2a1*, *Sox9*, and *Aggrecan* expression (Figs. 4E–G). TGFβ3-supplemented cultures achieved comparable chondrogenic differentiation as activin A (Figs. 4I, iii and iv, 4 J, and 4 K), validating that TGFβ is required at the initial stages of chondrogenesis (Kawaguchi et al., 2005; Diekman et al., 2010; Nakayama et al., 2003). Although FGF8 was shown to induce chondrogenesis (Abzhanov and Tabin, 2004; Bobick et al., 2007; Yu and Ornitz, 2008), it could not be used alone in our culture system because of poor cell-matrix attachment. This finding is consistent with the role of FGF8 in anchorage-independent cell growth and survival through interaction with the adaptor protein CRKL (Seo et al., 2009). Neither TGFβ3 nor FGF8 compensated for the noninductive effect of BMP4 (10 ng/ml) or further enhanced the progress of chondrocyte formation initiated by activin A when they were added from Day 5 to Day 15 of differentiation (Figs. 4L–N), reinforcing the stage-specific nature of TGFβ- and FGF-modulated chondrogenic induction.

We demonstrated that 5-day supplementation of activin A was sufficient to induce chondrogenesis in 15-day monolayer cultures (Figs. 5A–C), suggesting that ESCs acquired the capacity to become chondrocytes within the first 5 days of

Figure 6 BMP4 induced chondrogenic differentiation at 25 ng/ml while Wnt3a acted as a late inducer to amplify the effect of BMP4 (10 ng/ml). (A) Addition of Wnt3a at the onset of differentiation did not lead to Col2a1 expression even with further addition of factors like BMP4 (i, ii), but robust networks were formed in BMP4 (10 ng/ml) cultures further supplemented with Wnt3a as a secondary factor (iii, iv) (200×). (B) Compared to the treatment regime of adding Wnt3a ("W") at the onset of differentiation followed by BMP4 ("B"), Wnt3a supplementation of BMP4-treated cultures beginning on Day 5 of differentiation led to increases in *Col2a1*, *Sox9*, *Col10a, and Runx2* transcripts levels but not *Aggrecan*, regardless of the duration of BMP4 supplementation. "F1" = factor added at the onset of differentiation. Plotted values represent ratios of means ± SEM ($n \geq 3$). (C) Alcian blue staining reinforced the observation that Wnt3a acted as a late inducer of chondrogenesis. (D) BMP4 acted as an early chondrogenic inducer at 25 ng/ml as robust Col2a1 networks were generated regardless of a 15-day (i, iii, v) or 5-day (ii, iv, vi) supplementation regime. Further addition of (iii, iv) TGFβ3 or (v, vi) FGF8 did not have noticeable enhancing effects on Col2a1 network formation (200×). (E) qPCR analysis showed slight increases in *Col2a1* and *Sox9* in 5-day BMP4 (25 ng/ml) ("25B") cultures compared to a 15-day supplementation schedule. Additional treatment with TGFβ3 or FGF8 did not have significant enhancing effects on chondrogenic markers expression. Plotted values represent means ± SEM ($n \geq 3$). (F) Alcian blue staining confirmed the production of proteoglycans in cultures with BMP4 (25 ng/ml) under both (i–iii) 15-day and (iv–vi) 5-day supplementation schedules, while the presence of TGFβ3 (ii, v) and FGF8 (iii, vi) did not lead to increased proteoglycan production. Staining intensity in BMP4 (25 ng/ml) cultures was weaker than those supplemented with activin A.

differentiation in activin A-supplemented cultures. BMP4 has been shown to induce ESC chondrogenic differentiation (Kramer et al., 2000), and developing chondrocytes appear to undergo a BMP-dependent stage after initiation by TGFβ *in vivo* (Nakayama et al., 2003). We achieved BMP4-induced chondrogenic differentiation only when BMP4 concentration



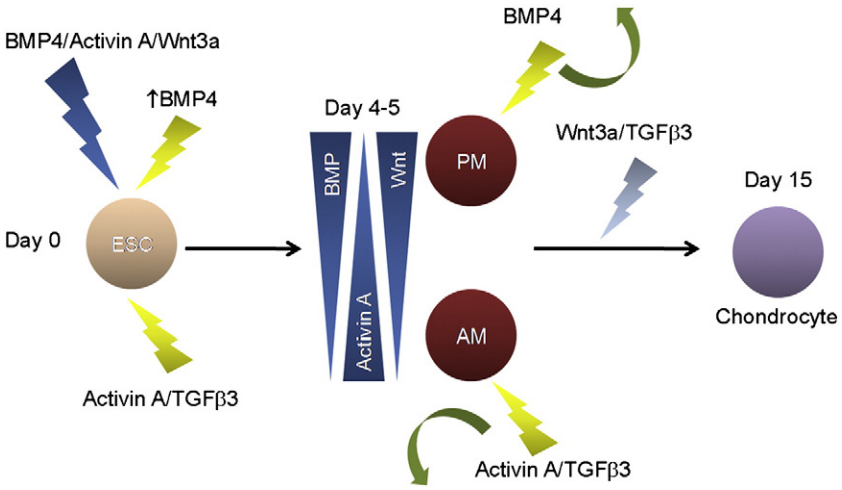


Figure 7 Schematic of our SF monolayer chondrogenic differentiation strategy. Supplementation of ESC cultures with BMP4 (10 ng/ml), activin A (30 ng/ml), or Wnt3a (100 ng/ml) on Day 0 of differentiation (dark blue thunderbolt) successfully induced mesoderm progenitors. Expression levels of mesoderm markers in SF monolayer cultures were in agreement with the notion that BMP4 and Wnt3a induced more posterior populations of mesoderm (PM) while activin A induced anterior mesoderm subsets (AM). Prolonged supplementation with activin A, TGFβ3 (10 ng/ml), or an increased concentration of BMP4 (25 ng/ml) (yellow thunderbolts) could induce chondrogenic differentiation after 15 days of culture. However, chondrogenic induction was not compromised when the duration of supplementation was shortened to 5 days (green arrows). Wnt3a was found to be a late inducer of chondrogenesis, and TGFβ3 could replace activin A on Day 5 of differentiation to promote the formation of articular cartilage (light blue thunderbolts).

increased from 10 to 25 ng/ml, with robust Col2a1 network formation (Fig. 6D, i and ii) and *Sox9* up-regulation (Fig. 6E), indicating that BMP4 also acted as an early inducer. However, BMP4 failed to up-regulate *Aggrecan* expression significantly when compared to activin A-supplemented cultures. Also, prolonged exposure to BMP4 (25 ng/ml) led to marginally lower transcript levels of *Col2a1* and *Sox9* but slightly higher *Runx2* expression compared to cultures with 5-day BMP4 treatment (Fig. 6E).

Activation of canonical Wnt signaling in nascent chondrocytes has been shown to block downstream chondrocyte development (Akiyama et al., 2004). Limb-bud and ESCs studies have concluded that Wnt3a is required during late-stage chondrocyte maturation, hypertrophy, and mineralization (Enomoto-Iwamoto et al., 2002; Kitagaki et al., 2003; Tamamura et al., 2005; Davis and Zur Nieden, 2008). Similarly, early Wnt3a treatment of our monolayer differentiation system generated mesoderm progenitors but did not promote chondrocyte formation. However, delayed Wnt3a supplementation of differentiation cultures initialized by activin A enhanced *Sox9*, *Aggrecan*, and *Runx2* expression (Fig. 5E), while Wnt3a treatment of BMP4 (10 ng/ml)-supplemented cultures resulted in robust Col2a1 network formation, superior expression of *Col2a1*, *Sox9*, *Col10a*, and *Runx2*, and more intense Alcian blue staining than in BMP4 alone (Figs. 6A–C). Since Wnt3a treatment of BMP4- and activin A-supplemented cultures appeared to up-regulate early and late chondrogenic markers, respectively, Wnt3a may have a compensatory prochondrogenic role in BMP4-containing cultures, while it promoted chondrocyte maturation in cells already induced by activin A.

In conclusion, we have developed a one-step strategy for generating monolayers of ESC-derived chondrogenic cells on collagen IV under chemically defined conditions. Our system recapitulated the published expression patterns of a plethora of mesoderm marker genes and confirmed the stage-specific nature of TGFβ-, BMP-, and Wnt-modulated chondrogenesis. The simplicity of our system facilitates the establishment of test cultures for HCI/HTS with minimal manipulation. The 2D nature of our system also provides a platform that permits easy visualization of changes in chondrogenic markers or reporter expression in knock-down/overexpression studies and in the identification of novel chondrogenic modulators.

Materials and methods

ESC maintenance and differentiation cultures

R1 ESCs were maintained as described in (Walker et al., 2007) and EBs were formed according to (Fok and Zandstra, 2005). N2B27 SF medium (Ying et al., 2003b) containing commercial N2 supplement (Gibco), X-Vivo™10 (Bio Whittaker) and CDM (Johansson and Wiles, 1995) were all supplemented with 1000 U/ml LIF (Chemicon), 1% GlutaMAX™ 1 (Gibco), and 0.1% β-mercaptoethanol (1000X, Gibco). LIF was replaced with activin A (R&D), BMP4 (R&D), or Wnt3a (Stem Cell Technologies) in SF differentiation medium. Wells were coated with gelatin (0.1%), gelatin (0.02%) + fibronectin (12.5 μg/ml), or collagen IV (250 μg/ml) (all from Sigma). For chondrogenic differentiation, 10^4cells/cm^2 were seeded onto collagen IV-coated wells and cultured for 15 days in SF differentiation medium supplemented with one growth factor or a combination of two factors (Supplementary Table 1). Serum monolayer cultures were established as described in (Nishikawa et al., 1998). All cultures were maintained at 37 °C under 5% CO_2.

Alcian blue staining

Cells were fixed with 95% ethanol for 3 h at -20 °C and washed with hydrochloric acid (HCl) (0.2 N) prior to staining with 0.1% Alcian blue (Sigma) in HCl at room temperature or 4 °C overnight. Cells were washed with HCl and imaged using a camera-mounted Leica MZ6 stereomicroscope at 12.5× magnification. Each figure represents 58% of the well.

Real-time quantitative PCR (qPCR)

Total RNA was isolated using the NucleoSpin® RNAII kit (Macherey-Nagel) treated with a DNA-free kit (Ambion). Random primers and Superscript® II reverse transcriptase (all from Invitrogen) were used to synthesize cDNA. Diluted cDNA was mixed with LightCycler® 480 DNA SYBR Green I Master reagent (Roche), primer mix (50 μM), and water. Standard curves were generated using genomic DNA or cDNA collected from EBs. Technical triplicates of each qPCR were carried out using the LightCycler® 480 Real-Time PCR System (Roche): 95 °C for 5 min; 45 cycles of (95 °C for 10 s, 60 °C for 10 s, and then 72 °C for 10 s). See Supplementary Table 2 for primer sequences. Measured transcript levels were normalized to *Eef1* or *GAPDH* and compared to Day 0 controls.

Statistical analysis

Statistical analysis was performed using the two-tail Student *t* test: significance between two test conditions, * ($P<0.05$), ** ($P<0.005$); significance between test condition and untreated SF control, ● ($P<0.05$), ●● ($P<0.005$), and significance between test condition and serum control, ▲ ($P<0.05$), ▲▲ ($P<0.005$). HCI and qPCR analyses were performed in triplicates. All experiments were independently replicated, as indicated by the *n* values in corresponding figure legends.

Supplementary materials related to this article can be found online at doi:10.1016/j.scr.2010.08.007.

Acknowledgments

We thank Dr. Kamal Garcha for advice and editing and Drs. Frank Beier, Cheryle Séguin, and Peter Zandstra for their insight. The work was supported by grants to W.L.S. from NSERC (RGPIN 293170-04) and the Canadian Arthritis Network. E.Y.L.W. was supported by Ontario Graduate Scholarships and W.L.S. is supported by a Canada Research Chair.

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